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Identification and codon reading properties of 5-cyanomethyl uridine, a new modified nucleoside found in the anticodon wobble position of mutant haloarchaeal isoleucine tRNAs

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ABSTRACT

Most archaea and bacteria use a modified C in the anticodon wobble position of isoleucine tRNA to base pair with A but not with G of the mRNA. This allows the tRNA to read the isoleucine codon AUA without also reading the methionine codon AUG. To understand why a modified C, and not U or modified U, is used to base pair with A, we mutated the C34 in the anticodon of *Haloarcula marismortui* isoleucine tRNA (tRNA^{Ile}₂) to U, expressed the mutant tRNA in *Haloferax volcanii*, and purified and analyzed the tRNA. Ribosome binding experiments show that although the wild-type tRNA^{Ile}₂ binds exclusively to the isoleucine codon AUA, the mutant tRNA binds not only to AUA but also to AUU, another isoleucine codon, and to AUG, a methionine codon. The G34 to U mutant in the anticodon of another *H. marismortui* isoleucine tRNA species showed similar codon binding properties. Binding of the mutant tRNA to AUG could lead to misreading of the AUG codon and insertion of isoleucine in place of methionine. This result would explain why most archaea and bacteria do not normally use U or a modified U in the anticodon wobble position of isoleucine tRNA for reading the codon AUA. Biochemical and mass spectrometric analyses of the mutant tRNAs have led to the discovery of a new modified nucleoside, 5-cyanomethyl U in the anticodon wobble position of the mutant tRNAs. 5-Cyanomethyl U is present in total tRNAs from euryarchaea but not in crenarchaea, eubacteria, or eukaryotes.

Keywords: 5-cyanomethyl uridine; tRNA modification; anticodon wobble position; codon reading properties; archaea

INTRODUCTION

Of the 16 four-codon boxes in the genetic code, the AUN box (N=U, C, A, or G) is unique in that three of the four codons AUU, AUC, and AUA specify one amino acid (isoleucine), whereas the fourth codon, AUG, specifies another amino acid (methionine). This three-to-one distribution of codons within a four-codon box is different from essentially all other codon boxes in which all four codons either specify the same amino acid or are split two to two, codons ending in pyrimidines specifying one amino acid, and codons ending in purines specifying a different amino acid (Khorana 1968; Nirenberg 1968).

Archaea and bacteria use two different isoleucine tRNAs (tRNA^{Ile}₁ and tRNA^{Ile}₂) to read the three isoleucine codons. tRNA^{Ile}₁ with the anticodon GAU reads AUU and AUC following the Wobble Hypothesis (Crick 1966), whereas tRNA^{Ile}₂ reads the remaining isoleucine codon AUA without misreading the methionine codon AUG (Harada and Nishimura 1974; Köhrer et al. 2008; Ikeuchi et al. 2010; Mandal et al. 2010). Most interestingly, in both kingdoms, tRNA^{Ile}₂ has almost always C*AU as the anticodon sequence, C*, a modified C in the anticodon wobble position, being agmatidine in archaea (Ikeuchi et al. 2010; Mandal et al.

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2010) and lysidine in bacteria (Muramatsu et al. 1988; Grosjean and Björk 2004). Thus, C* can form a base pair with A of the AUA but not with G of the AUG codon. This raises the question of why archaea and bacteria have evolved a mechanism to use a modified C instead of a U or a modified U to base pair exclusively with A.

In an attempt to answer the preceding question, we mutated the C in the anticodon of the *Haloarcula marismortui* tRNA₂^{Ile} gene to U (U34 mutant), expressed the mutant tRNA in *Haloferax volcanii*, and purified it. The mutant tRNA was found to be a poorer substrate for the haloarchaeal isoleucyl-tRNA synthetase (IleRS) in vivo and in vitro. The mutant tRNA was, however, a substrate for *Escherichia coli* IleRS, and this allowed us to aminoacylate it with isoleucine and study its codon reading properties using *H. marismortui* ribosomes. In contrast to wild-type Ile-tRNA₂^{Ile}, which binds only to the AUA codon, the U34 mutant tRNA binds not only to AUA but also to AUU, another isoleucine codon, and AUG, a methionine codon. To determine whether the U34 mutant derived from *H. marismortui* tRNA₁^{Ile} species would have similar coding properties, we also mutated G34 to U34 in the anticodon of tRNA₁^{Ile}. The U34 mutant of tRNA₁^{Ile} also bound to AUA, AUU, and AUG.

Analyses of the U34 mutant tRNAs showed that U34 in the anticodon wobble position is modified to 5-cyanomethyl U (cnm⁵U), a new modified nucleoside. We, therefore, investigated (1) whether some other naturally occurring tRNAs in *H. volcanii* contain this modified nucleoside; and (2) how widespread the occurrence of this modified nucleoside is in tRNAs from other archaeal organisms and from other kingdoms. Mass spectrometric analyses of nucleosides present in total tRNAs indicate that cnm⁵U is present in total tRNA from haloarchaea and *Methanococcus maripaludis* but not in total tRNA from *Sulfolobus solfataricus*, *Saccharomyces cerevisiae*, and *Escherichia coli*, suggesting its possible presence only in tRNAs from euryarchaea.

RESULTS

Expression of *H. marismortui* wild-type and mutant isoleucine tRNAs in *H. volcanii* and growth phenotype of transformants

Previously, we described the purification of *H. marismortui* tRNA₂^{Ile} and showed that it contained agmatidine in the anticodon wobble position and bound to the isoleucine codon AUA but not to the methionine codon AUG (Mandal et al. 2010). In this work, we mutated the C in the anticodon wobble position of the tRNA₂^{Ile} gene to U (Fig. 1A) and ex-

pressed the U34 mutant tRNA in *H. volcanii*. In a parallel experiment, the G34 in the anticodon wobble position of tRNA₁^{Ile} was also mutated to U34 (Fig. 1B) and expressed in *H. volcanii*.

H. volcanii cells were transformed with pWL201-derived expression vectors carrying either the *H. marismortui* wild-type tRNA₂^{Ile} gene, mutant tRNA₂^{Ile} gene, wild-type tRNA₁^{Ile} gene, or the mutant tRNA₁^{Ile} gene; the transformants were plated on media containing 4 μg/mL of mevinolin. Colonies of good size appeared 5–7 d after plating; however, transformants containing either of the mutant tRNA genes were always delayed by ~2 d. Also, although plasmids carrying the empty pWL201 vector or either of the wild-type tRNA genes yielded ~10⁷ transformants/μg of plasmid DNA, those carrying either of the mutant tRNA genes yielded only 10⁵–10⁶ transformants/μg of plasmid DNA. Additionally, when individual freshly isolated colonies of similar size were used to inoculate liquid media containing mevinolin and growth was monitored over a period of several days, there was a consistent lag of 1–2 d in growth of transformants carrying the mutant tRNA genes (Fig. 1C,D), although the transformants eventually grow at a rate similar those carrying the wild-type tRNA genes. Taken together, these findings suggest that *H. volcanii* cells expressing the mutant tRNAs are less fit and require a phase of adaptation.

Purification of U34 mutants of tRNA₂^{Ile} and tRNA₁^{Ile}

As for the wild-type tRNAs, the mutant tRNAs were purified by hybrid selection using a biotinylated DNA oligonucleotide bound to streptavidin-sepharose, followed by native

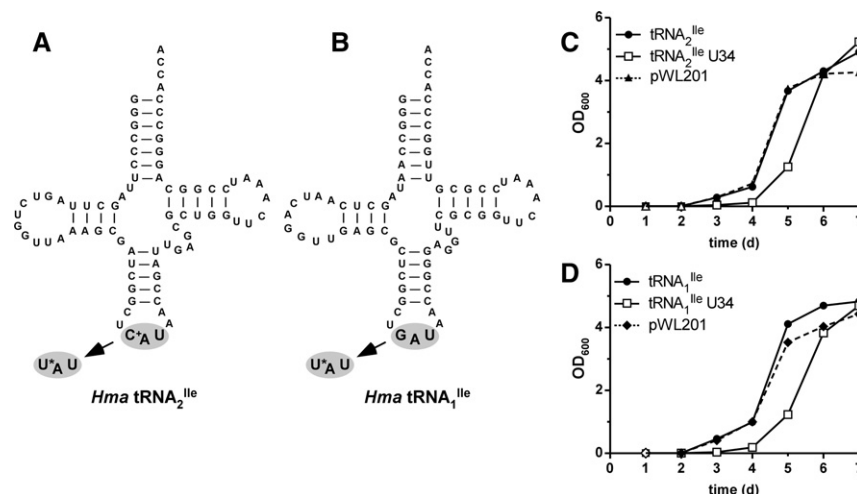


FIGURE 1. *H. marismortui* isoleucine tRNA mutants containing 5-cyanomethyl uridine at the wobble position. Cloverleaf structures of tRNA₂^{Ile} (A) and tRNA₁^{Ile} (B) from *H. marismortui*. The anticodon changes at the wobble position from C⁺ to U* and G to U*, respectively, are indicated. (C⁺) agmatidine; (U*) 5-cyanomethyl uridine (cnm⁵U). Growth of *H. volcanii* cells transformed with plasmids for overexpression of *H. marismortui* tRNA₂^{Ile} wild-type and the tRNA₂^{Ile} U34 mutant (C), and *H. marismortui* tRNA₁^{Ile} wild-type and the tRNA₁^{Ile} U34 mutant (D). The empty vector (pWL201) is shown as a control in C and D.

polyacrylamide gel electrophoresis (Suzuki and Suzuki 2007; Mandal et al. 2010). Figure 2A shows that the purified mutant tRNA₂^{Ile} is essentially homogeneous and consists of a major band and a minor band (Fig. 2A, left panel, lane 3), both of which hybridize to a DNA oligonucleotide probe complementary to *H. marismortui* tRNA₂^{Ile} (Fig. 2A, right panel, lane 3). The yield of purified tRNA from a 12-L culture of *H. volcanii* was about 4 A₂₆₀ units and represented ~0.4% of total tRNA. The U34 mutant of *H. marismortui* tRNA₁^{Ile} was purified similarly and also yielded a homogeneous product (Supplemental Fig. S1).

Additional evidence for homogeneity of the purified tRNA was derived from partial RNase T1, RNase A, and RNase U2

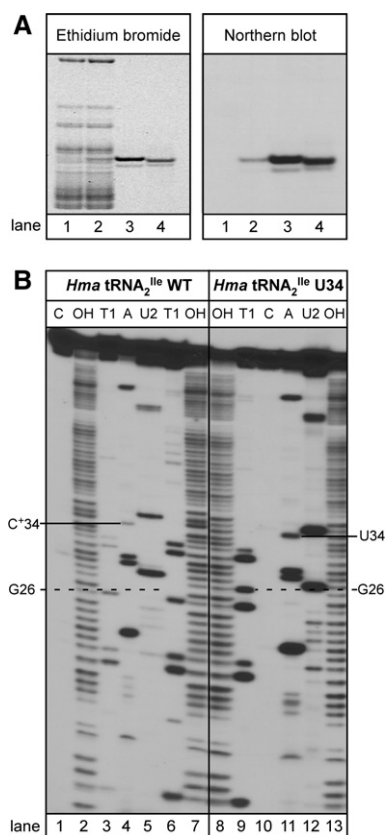


FIGURE 2. Purification and characterization of the *H. marismortui* tRNA₂^{Ile} U34 mutant. (A) Overexpression and purification of the *H. marismortui* tRNA₂^{Ile} U34 mutant. Native PAGE analysis of total tRNA from *H. volcanii* (lane 1), total tRNA from *H. volcanii* overexpressing the *H. marismortui* tRNA₂^{Ile} U34 mutant (lane 2), and purified *H. marismortui* tRNA₂^{Ile} U34 mutant (lane 3). Purified *H. marismortui* wild-type tRNA₂^{Ile} is shown as a control (lane 4). One-tenth A₂₆₀ unit of total tRNA and 0.01 A₂₆₀ of purified tRNA were applied per lane. tRNAs are visualized by ethidium bromide staining (left panel) or Northern blot analysis using a probe specific for *H. marismortui* tRNA₂^{Ile} (right panel). (B) Characterization of purified *H. marismortui* wild-type and U34 mutant tRNA₂^{Ile}. The homogeneity of purified tRNAs was confirmed by partial RNase T1 (T1 lanes), RNase A (A lanes), RNase U2 (U2 lanes) digests of 5'-³²P-labeled tRNA. Two different concentrations of RNase T1 were used in lanes 3 and 6. (Lane OH) partial alkali hydrolysis; (Lane C) undigested tRNA control. ³²P-labeled fragments were separated by denaturing PAGE and visualized by autoradiography.

digests of 5'-³²P-labeled tRNA (Donis-Keller et al. 1977; Simoncsits et al. 1977; Lockard et al. 1978). All the ³²P-labeled bands present in the RNase T1 digest were as expected for the mutant tRNA and were essentially the same as that for the wild-type tRNA (Fig. 2B, cf. lanes 3 and 6 to lane 9). The only exception was an RNase T1 cut at position 26 of the mutant tRNA, which is absent in RNase T1 digests of the wild-type tRNA. This is most likely due to a partial lack of modification of G to N²N²-dimethyl G at position 26 in the mutant tRNA. The cleavage patterns seen in digests with RNase A and RNase U2 are also essentially identical between the mutant and the wild-type tRNAs and are, as expected, based on the tRNA sequence (Fig. 2B, cf. lane 4 to lane 11, lane 5 to lane 12). Because partial digestions with RNases A and U2 were carried out in the absence of urea, digestion with these enzymes is more limited compared to digestion with RNase T1 in the presence of 7 M urea. Basically, similar results were obtained for RNase T1 and RNase A digests of 5'-³²P-labeled wild-type and U34 mutant of tRNA₁^{Ile} (Supplemental Fig. S2).

The U34 mutant tRNA₂^{Ile} and tRNA₁^{Ile} overexpressed in *H. volcanii* are poor substrates for aminoacylation by *H. volcanii* IleRS

To determine the aminoacylation status of the *H. marismortui* mutant tRNAs, which are overexpressed in *H. volcanii*, we isolated total tRNA from a small scale culture of *H. volcanii* under acidic conditions, separated tRNAs from aminoacyl-tRNAs on an acid urea polyacrylamide gel, and detected wild-type and mutant *H. marismortui* tRNA₂^{Ile} by RNA blot hybridization (Varshney et al. 1991; Köhrer and RajBhandary 2008). Both the mutant and the wild-type tRNA₂^{Ile} were aminoacylated rather poorly (20%–25% and 25%–30%, respectively), based on phosphorimager analysis of the bands, in *H. volcanii* (Fig. 3A, top panel). For the U34 mutant derived from tRNA₁^{Ile}, the corresponding numbers were ~60% for the mutant and >90% for the wild-type tRNA (Fig. 3B, top panel). Control experiments using *H. volcanii* tRNA₂^{Ile} and tRNA₁^{Ile} probes on the same blots show that the endogenous wild-type *H. volcanii* tRNA₂^{Ile} and tRNA₁^{Ile} are aminoacylated essentially quantitatively (Fig. 3A,B, bottom panels).

In vitro aminoacylation of the purified *H. marismortui* mutant tRNA₂^{Ile} and tRNA₁^{Ile} using IleRS present in S10, S30, or S100 extracts of *H. volcanii* showed that the mutant tRNAs were poor substrates for the archaeal IleRS (data not shown). The mutant tRNAs could, however, be aminoacylated essentially quantitatively with ³H-Ile using an excess of purified *E. coli* IleRS (Supplemental Fig. S3).

Codon-reading properties of the U34 mutant tRNA₂^{Ile} and tRNA₁^{Ile}

Purified wild-type and mutant tRNA₂^{Ile} and mutant tRNA₁^{Ile} were aminoacylated with ³H-Ile using *E. coli* IleRS, and the

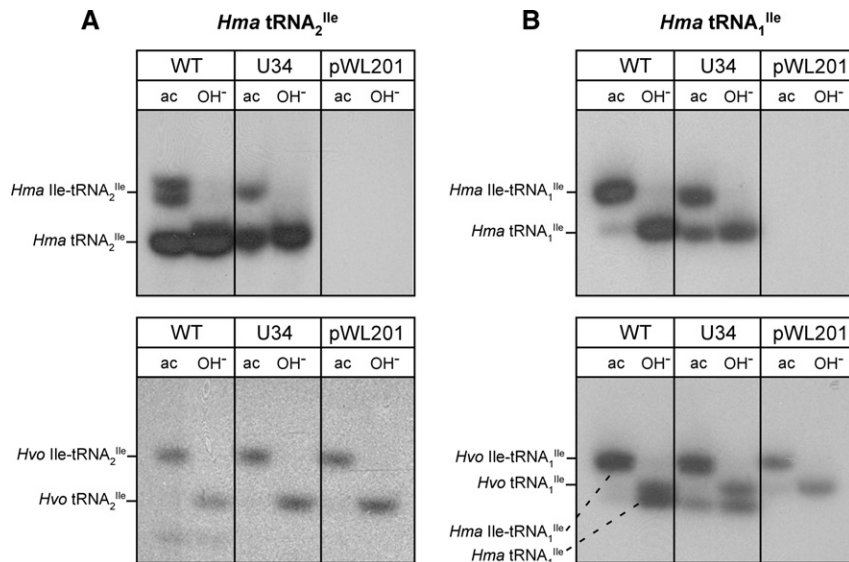


FIGURE 3. State of in vivo aminoacylation of *H. marismortui* wild-type and U34 mutant tRNA₂^{Ile} (A) and wild-type and U34 mutant *H. marismortui* tRNA₁^{Ile} (B). Total tRNA was isolated under acidic conditions and analyzed by acid urea PAGE followed by Northern hybridization using probes specific for *H. marismortui* tRNA₂^{Ile} (A, top panel) and *H. marismortui* tRNA₁^{Ile} (B, top panel), respectively. The same blots were stripped and rehybridized using probes specific for the endogenous *H. volcanii* tRNA₂^{Ile} (A, bottom panel) and *H. volcanii* tRNA₁^{Ile} (B, bottom panel). Note that the *H. volcanii* tRNA₁^{Ile}-specific probe also picks up the overexpressed *H. marismortui* tRNA₁^{Ile} because of the high levels of overexpression of *H. marismortui* tRNAs and close sequence similarity between the overexpressed and the endogenous tRNA₁^{Ile}. (ac) tRNA isolated under acidic conditions; (OH⁻) tRNA after deacylation by base-treatment.

³H-Ile-tRNAs were used for studying oligonucleotide-dependent binding to *H. marismortui* ribosomes. The oligonucleotides used were AUGAUA, AUGAUC, AUGAUU, and AUGAUG. Figure 4 shows the results. As previously published (Mandal et al. 2010), the wild-type Ile-tRNA₂^{Ile} binds to AUA but not to AUU, AUC, or AUG (Fig. 4A). In contrast, the mutant tRNA₂^{Ile} binds best to AUA, but it also binds to AUU, another isoleucine codon, and to AUG, a methionine codon (AUA>AUG>AUU) (Fig. 4B). The codon-dependent binding of the tRNA to the ribosome is dependent in all cases on concentration of the oligonucleotide. The codon binding properties of mutant tRNA₁^{Ile} are the same as those of the mutant tRNA₂^{Ile} (AUA>AUG>AUU) (Fig. 4C).

Analysis of nucleotide in the anticodon wobble position of the mutant tRNA

In view of the codon binding properties of the U34 mutant tRNAs, a key question of interest is whether U34 in the mutant tRNA is modified in *H. volcanii*; if so, what is the nature of the modification? For this, we partially digested the mutant tRNA₂^{Ile} randomly with alkali, labeled the 5'-hydroxyl groups of the fragments generated with ³²P using T4 polynucleotide kinase, and separated the 5'-³²P-labeled fragments on a denaturing polyacrylamide gel (Stanley and Vassilenko 1978; Gupta and Randerath 1979; Kuchino et al. 1979; RajBhandary 1980). Figure 5A shows part of the pattern obtained for the

wild-type and the mutant tRNAs. The digest of wild-type tRNA shows a pronounced shift in the electrophoretic mobility of fragments, which are produced by cleavage of phosphodiester bonds between nucleotides 33/34 and 34/35, and which differ by one nucleotide. This is due to the presence of the positively charged agmatidine at position 34 (Mandal et al. 2010). As expected, such a shift in mobility between neighboring fragments is not observed in digests of the U34 mutant tRNA.

For identification of the 5'-terminal nucleotides of the fragments, the ³²P-labeled bands corresponding to nucleotides 32–38 in the anticodon loop of the wild-type and mutant tRNA were eluted from the gel, cleaved with nuclease P1, and the ³²P-labeled 5'-terminal nucleotide of each fragment was identified by thin layer chromatography (Nishimura 1979; Silberklang et al. 1979). Figure 5B shows the two-dimensional thin layer chromatography pattern obtained for U34 in the mutant tRNA. The 5'-³²P-labeled nucleotide migrates very close to, but not identical with, pU. Thus, U34 in the mutant tRNA contains a base modification. A similar analysis of the U34 mutant derived from *H. marismortui* tRNA₁^{Ile} shows that U34 in this mutant tRNA also carries the same base modification (Fig. 5C).

Mass spectrometric identification of the modified U as 5-cyanomethyl U (cm⁵U)

To identify nucleosides that differed between wild-type and the U34 mutant *H. marismortui* tRNA₂^{Ile}, purified tRNA₂^{Ile} was enzymatically hydrolyzed, and the nucleosides were resolved by reversed-phase HPLC. Comparison of HPLC profiles showed the presence of a nucleoside present exclusively in the mutant tRNA₂^{Ile} (retention time 7.9 min) (Fig. 6, vertical arrow). Subsequent analysis of both samples by liquid chromatography-coupled quadrupole time-of-flight mass spectrometry (LC-QTOF) revealed a species with *m/z* 284.0872 (Fig. 7A), again only in the mutant tRNA. In the same high-resolution spectrum, we also identified an ion with *m/z* 152.0451, which is consistent with the loss of ribose from a precursor species with *m/z* 284.0872 (Fig. 7A). Similar analysis of a chemically synthesized 5-cyanomethyl-2'-deoxyuridine standard (Fig. 8B; Sakata et al. 1980; Kuwahara et al. 2006) revealed an ion with an identical *m/z* of 152.0451 (Fig. 7C).

The mass spectral data for the modified ribonucleoside identified in this study suggested a chemical formula of

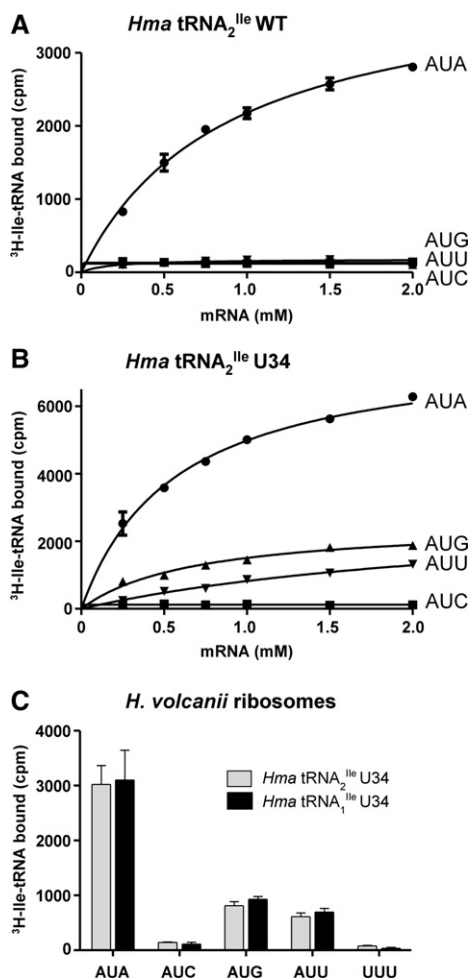


FIGURE 4. Ribosome binding of U34 mutants of *H. marismortui* tRNA₂^{Ile} and tRNA₁^{Ile}. Template-dependent binding of purified ³H-Ile-tRNA₂^{Ile} wild-type (A) and ³H-Ile-tRNA₂^{Ile} U34 (B) to ribosomes isolated from *H. marismortui*. Oligonucleotides used were AUGAUA, AUGAUG, AUGAUG, and AUGAUU. (C) Binding of purified ³H-Ile-tRNA₂^{Ile} U34 and ³H-Ile-tRNA₁^{Ile} U34 to ribosomes isolated from *H. volcanii*; the mRNA concentration was 1 mM; the AUGUUU and “no mRNA” controls are also shown in C. Results from three independent experiments are shown.

C₁₁H₁₃N₃O₆ (calculated m/z 284.0877), consistent with a U modified with methylene and cyano functional groups. Comparison of the UV absorption spectra of the modified U (isolated by HPLC), thymidine, and the synthetic 5-cyanomethyl-2'-deoxyuridine showed that they all had a λ_{\max} at 267 nm (Supplemental Fig. S4), suggesting that the cyano group is not attached directly to the ring, and the modified U most likely has a $-\text{CH}_2-\text{C}\equiv\text{N}$ side chain. Since most tRNA anticodon wobble modifications on U occur at the 5-position of the base (Yokoyama and Nishimura 1995), one candidate structure for the modified U is 5-cyanomethyl uridine (cnm⁵U) (Fig. 8A).

This structure was confirmed by tandem mass spectrometric analysis of the modified U in comparison with the synthetic 5-cyanomethyl-2'-deoxyuridine. As shown in Figure

7B and D, both the modified U and 5-cyanomethyl-2'-deoxyuridine were subjected to pseudo-MS³ fragmentation analysis by LC-QTOF. In this analysis, the nucleoside is deglycosylated in-source, and the resulting protonated base was selected by Q1 for further fragmentation by collision induced dissociation (CID) in the collision cell. In the high-resolution tandem mass spectra thus obtained, nucleobases from both the modified U (Fig. 7B) and the synthetic 5-cyanomethyl-2'-deoxyuridine (Fig. 7D) generated identical fragmentation patterns with m/z values of 125, 82, and 54, which are consistent with the proposed fragmentation pathway shown in Figure 7E. If the cyanomethyl group was present at position 5 of U, and not position 6, then CID fragmentation should also produce the allene species 5 (m/z 39) shown in Figure 7E. Although this species was not apparent by QTOF analysis due to low sensitivity, analysis of the protonated base ion by in-source fragmentation of the modified U and the 5-cyanomethyl-2'-deoxyuridine standard by tandem triple-quadrupole (QqQ) mass spectrometry revealed a species with m/z 39 for both nucleosides (Fig. 7B,D, insets). Altogether, our data identify cnm⁵U as the unique modified U in the mutant *H. marismortui* tRNA₂^{Ile}. A similar mass spectrometric analysis of purified mutant *H. marismortui* tRNA₁^{Ile} also confirmed the presence of cnm⁵U in this tRNA (data not shown).

cnm⁵U is present in total tRNAs from haloarchaea and *M. maripaludis* but not in tRNAs from *S. solfataricus*, *S. cerevisiae*, and *E. coli*

The identification of cnm⁵U as a new modified nucleoside in mutant *H. marismortui* tRNA₂^{Ile} and tRNA₁^{Ile} expressed in *H. volcanii* raised the question of whether it is present in total tRNA from *H. volcanii* and from other organisms. To identify cnm⁵U in endogenous tRNAs in *H. volcanii*, we analyzed total tRNA digests with or without expression of the *H. marismortui* U34 mutant tRNA₂^{Ile} by QTOF. To reduce background and improve detection, ribonucleosides in total tRNA digests were separated by HPLC, and fractions eluting in the region of cnm⁵U were collected and subjected to LC-QTOF. We found cnm⁵U in total tRNA with or without expression of the U34 mutant *H. marismortui* tRNA₂^{Ile}, suggesting the presence of cnm⁵U in endogenous tRNAs in *H. volcanii* (data not shown). We also developed a multiple reaction-monitoring (MRM) method based on the fragmentation information from the LC-QTOF analysis of 5-cyanomethyl-2'-deoxyuridine and cnm⁵U, using a tandem triple-quadrupole mass spectrometer (Fig. 9A); and using this approach, we observe a distinct peak corresponding to cnm⁵U in total tRNA isolated from wild-type *H. volcanii* cells (Fig. 9B).

We subsequently analyzed total tRNA from *Halobacterium salinarum*, *H. marismortui*, *M. maripaludis*, *S. solfataricus*, *S. cerevisiae*, and *E. coli* for the presence of cnm⁵U. In the case of *H. salinarum*, *H. marismortui*, *M. maripaludis*, and *S. solfataricus*, total tRNA digests were directly analyzed using the

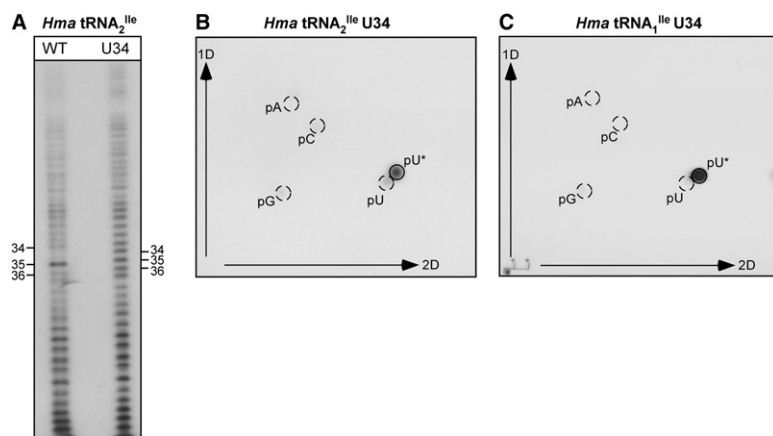


FIGURE 5. 5'-³²P-labeling of random alkali fragments (A), and 2D-TLC analysis of the wobble base nucleotide in U34 mutants of *H. marismortui* tRNA₂^{Ile} (B) and tRNA₁^{Ile} (C). Purified tRNAs were cleaved randomly with alkali; fragments were 5'-end labeled with ³²P and separated by gel electrophoresis. The 5'-end nucleotide of the fragment corresponding to U*34 was analyzed by nuclease P1 digestion and 2D-TLC. Nonradioactive markers pA, pC, pG, and pU were visualized by UV-shadowing and are indicated by circles. (pU*) position of *cnm*⁵U.

MRM method (Fig. 9C–F); in the case of *S. cerevisiae* and *E. coli*, HPLC fractions eluting at the *cnm*⁵U retention time were collected and the prepurified fractions were subjected to LC-QTOF analysis (Fig. 9G,H). In all cases, purified *cnm*⁵U from the U34 mutant tRNA₂^{Ile} and total tRNAs from *H. volcanii* were used as positive control. Within the detection limits of the instrument, we found *cnm*⁵U in total tRNA from haloarchaea and *M. maripaludis* but not from *S. solfataricus*, *S. cerevisiae*, and *E. coli*.

DISCUSSION

To answer the question why most bacterial and archaeal isoleucine tRNAs use lysidine or agmatidine in the anticodon wobble position to base pair exclusively with A but not with G, we have reported here the expression, purification, and analysis of the codon binding properties of mutants of *H. marismortui* tRNA₂^{Ile} and tRNA₁^{Ile}, in which the anticodon wobble position has been mutated to U. The U34 mutants of *H. marismortui* tRNA₂^{Ile} and tRNA₁^{Ile} bind not only to the isoleucine codons AUA and AUU but also to the methionine codon AUG. Binding to AUG could lead to misreading of AUG and insertion of isoleucine in place of methionine into proteins. The extent of misreading of the AUG codon in *H. volcanii* would, however, depend upon several factors, including the extent of aminoacylation of the mutant tRNA, its concentration in vivo, its relative affinity for the AUG codon on the ribosome, and competition by the endogenous *H. volcanii* elongator Met-tRNA^{Met}, which normally binds to the AUG codon. The findings that transformants carrying the genes for the U34 mutants of tRNA₂^{Ile} or tRNA₁^{Ile} have a growth disadvantage compared with those carrying the wild-type tRNA₂^{Ile} or tRNA₁^{Ile} (Fig. 1C,D) suggest that misreading of

AUG also occurs in vivo. This would explain why nearly all archaea and bacteria use a modified C and not U or a modified U in the anticodon wobble position to read the isoleucine codon AUA. Although agmatidine and lysidine, both modified derivatives of C, base pair specifically with A of AUA, similar derivatives of U have so far not been found in nature. It is likely that such U derivatives would have tautomeric structures in which both the N³ and O⁴ could act as H-bond acceptors and, therefore, be incapable of forming stable base pairs with A on the ribosome.

Our results do not, however, rule out the possibility that some archaeal or bacterial organism will use U or a different modified U in the anticodon wobble position to read the isoleucine codon AUA without also reading the methionine codon AUG. For example, most eukaryotic organisms encode an isoleucine tRNA (tRNA₂^{Ile}) that has a U modified to Ψ in the anticodon wobble position (Senger et al. 1997). The Ψ in this tRNA is thought to base pair with A of the AUA codon but not with G of the AUG codon. Also, it is known that two archaeal organisms, a nanoarchaeon and a korarchaeon (Waters et al. 2003; Randau et al. 2005; Elkins et al. 2008), and some bacteria including *Mycoplasma mobile*, *Bifidobacterium adolescentis*, and a mutant strain of *B. subtilis* encode tRNA^{Ile} with a UAU in the anticodon (Fabret et al. 2011). It has been shown that the U in the anticodon wobble position of tRNA^{Ile} from *M. mobile* and the mutant tRNA^{Ile} from *B. subtilis* is not modified, and these tRNAs read the isoleucine codon AUA without misreading the methionine

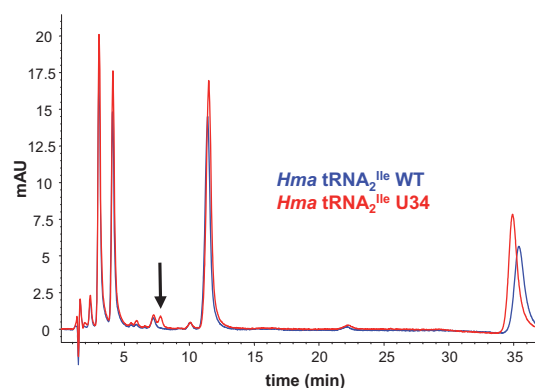


FIGURE 6. Identification of a unique ribonucleoside species in the U34 mutant tRNA₂^{Ile}. Purified *H. marismortui* wild-type and the U34 mutant thereof were digested to ribonucleosides and resolved by HPLC. The UV (260 nm) absorption patterns of the two digests were overlaid, and a unique peak was identified in the mutant sample, highlighted by the arrow.

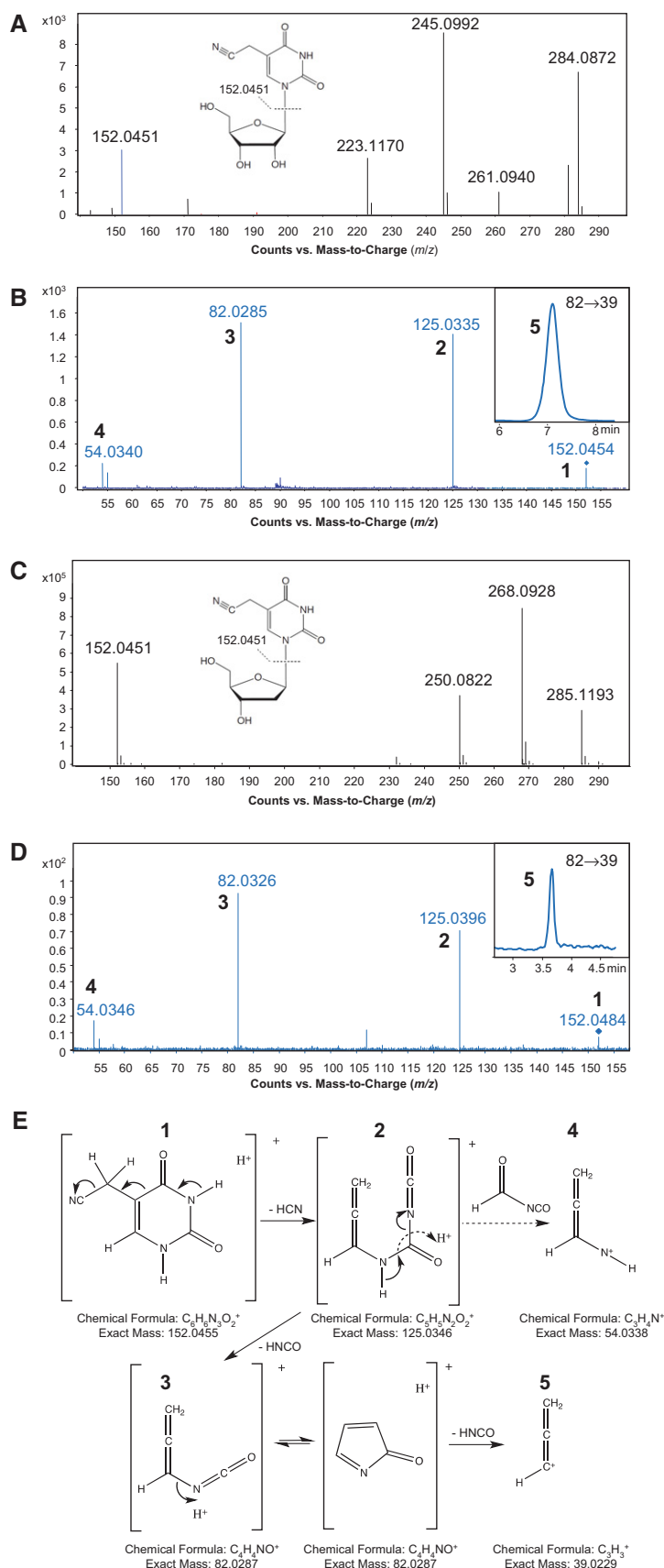


FIGURE 7. (Legend on next page)

codon AUG to any significant extent in vivo and in vitro (Köhler et al. 2013; Taniguchi et al. 2013). It is possible that in these few organisms, the ribosomes and the tRNA^{Ile} sequences have co-evolved in such a way that U or a modified U base pairs much better with A than with G.

Characterization of the U34 mutants of *H. marismortui* tRNA₂^{Ile} and tRNA₁^{Ile} led to the discovery of cm⁵U, a new modified nucleoside in *H. volcanii*. Mass spectrometric analysis of nucleosides derived from total tRNAs isolated from *H. volcanii*, *H. salinarum*, *H. marismortui*, and *M. maripaludis* has shown that this modified nucleoside is also present in endogenous tRNAs from these organisms but not in total tRNAs isolated from *S. solfataricus* (a crenarchaeon), *S. cerevisiae*, or *E. coli*. Thus, cm⁵U may be present mostly in tRNAs from euryarchaea.

Ribosome binding experiments with the U34 mutant of *H. marismortui* tRNA₂^{Ile} show that it binds to AUA, AUU, and AUG (AUA>AUG>AUU) with minimal binding, if any, to AUC (Fig. 4B,C). The corresponding mutant of tRNA₁^{Ile} also binds to AUA, AUU, and AUG but not to AUC (Fig. 4C). These results suggest that cm⁵U in the anticodon wobble position of a tRNA forms base pairs with U, A, or G on the mRNA.

The codons of a four-codon box in the genetic code Table are, in general, read by at least two tRNAs. For example, in eukaryotes a tRNA with inosine in the anticodon wobble position reads codons ending in U, C, and A, whereas another tRNA with C in the anticodon wobble position reads codons ending in G (Björk 1998). In contrast, in bacteria such as *E. coli*, a tRNA with 5'-carboxymethoxy U (cmo⁵U) in the anticodon wobble position reads codons ending in U, A, and G, whereas another tRNA with G in the anticodon wobble position reads codons ending in U and C (Murao et al. 1970; Yokoyama and Nishimura 1995). Similarly, in *B. subtilis*, a tRNA with 5'-methoxy U (mo⁵U) reads codons ending in U, A, and G, and a tRNA with G in the anticodon reads codons ending in U and C (Murao et al. 1976; Yokoyama

and Nishimura 1995). Information on the codon recognition patterns and properties of archaeal tRNAs is, however, quite limited. Except for the classical work of Gupta on nucleotide sequences of *H. volcanii* tRNAs and identification of many of the modified bases in these tRNAs (Gupta 1984, 1986) and identification of genes encoding many of the tRNA modifying enzymes using bioinformatic analyses (Phillips and de Crecy-Lagard 2011), there has been very little work done with purified tRNAs from archaea and on the codon recognition properties of archaeal tRNAs. The results we have obtained with two mutant haloarchaeal tRNAs containing cm^5U in the anticodon wobble position suggest that the codon recognition properties of cm^5U , which base pairs with U, A, or G, parallel those of cmo^5U in *E. coli* and mo^5U in *B. subtilis*. With the knowledge that other tRNAs in *H. volcanii* contain cm^5U (this work), it becomes important now to purify and identify these tRNAs and establish their codon recognition properties using *H. volcanii* ribosomes.

Finally, in spite of the fact that *H. volcanii* tRNAs have been among the best studied among the archaea in terms of nucleotide sequences and base modifications, our recent work on two haloarchaeal tRNAs, $\text{tRNA}_2^{\text{lle}}$ and a mutant derived from it, led to the discovery of two new modified nucleosides, agmatidine and 5-cyanomethyl uridine in *H. volcanii* and other haloarchaea. With so many other archaeal organisms, whose tRNAs have not been purified or studied to any extent, most likely there are many modified nucleosides with interesting structures and functions that remain to be discovered (Phillips and de Crecy-Lagard 2011). The methods for tRNA purification and analysis used here combined with the increased power and sophistication of mass spectral techniques and instrumentation should significantly facilitate this discovery.

MATERIALS AND METHODS

General

Strain *H. volcanii* WFD11 and plasmids pUCsptProM and pWL201 (Lam and Doolittle 1989; Nieuwlandt and Daniels 1990) were kindly provided by Drs. John R. Palmer and Charles J. Daniels (Department of Microbiology, Ohio State University). *H. marismortui* ATCC 43,049 was kindly provided by Dr. Peter Moore (Department of Chemistry, Yale University). The *E. coli* strains used in this work,

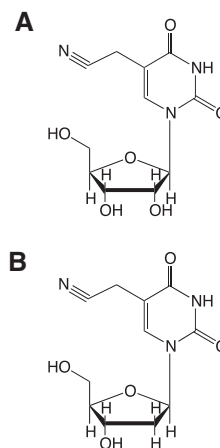


FIGURE 8. Chemical structures of 5-cyanomethyl uridine (A) and 5-cyanomethyl-2'-deoxyuridine (B).

E. coli XL1-blue and *E. coli* GM2163, have been described before (Ramesh and RajBhandary 2001). General manipulations of *H. marismortui*, *H. volcanii*, and *E. coli* were performed according to standard procedures (DasSarma and Fleischmann 1995; Sambrook and Russell 2001). RNase T1, RNase A, RNase U2, nuclease P1, and snake venom phosphodiesterase I were from Sigma; T4 polynucleotide kinase (T4-PNK), antarctic phosphatase, calf intestinal phosphatase, and inorganic pyrophosphatase were from New England Biolabs; DNA and RNA oligonucleotides were from IDT; and oligonucleotides used for cloning, detection, and purification of tRNAs are listed in Supplemental Table S1.

Cloning and overexpression of *H. marismortui* $\text{tRNA}_2^{\text{lle}}$ and $\text{tRNA}_1^{\text{lle}}$ (WT and the U34 mutant)

The gene for *H. marismortui* $\text{tRNA}_2^{\text{lle}}$ was first cloned into the vector pUCsptProM (Ramesh and RajBhandary 2001). For this, the gene for *H. marismortui* $\text{tRNA}_2^{\text{lle}}$ was PCR amplified from *H. marismortui* genomic DNA and inserted into the *Xba*I and *Bam*HI sites of plasmid pUCsptProM downstream from the tRNA^{Lys} promoter to generate pUCHmalle2WT. The C34 to U34 mutation in the anticodon of $\text{tRNA}_2^{\text{lle}}$ was generated by QuikChange mutagenesis (Stratagene) using pUCHmalle2WT as template. A *Hind*III/*Eco*RI fragment containing the wild-type $\text{tRNA}_2^{\text{lle}}$ gene or its U34 mutant, including the tRNA^{Lys} promoter, was subcloned into the shuttle plasmid pWL201 to generate pWLHmalle2WT and pWLHmalle2MUT. Similarly, pWLHmalle1WT and pWLHmalle1MUT carrying the *H. marismortui* wild-type $\text{tRNA}_1^{\text{lle}}$ gene or its U34 mutant were generated. All initial cloning was done in *E. coli* XL-1 blue. After confirming the sequences of the desired wild-type and mutant tRNA genes, the respective plasmids were used to transform *E. coli* GM2163 (*dam*⁻). Plasmid DNA isolated from this strain was then used to transform *H. volcanii* as described (DasSarma and Fleischmann 1995; Ramesh and RajBhandary 2001). Transformants were grown in *H. volcanii* medium containing 4 $\mu\text{g}/\text{mL}$ mevinolin (US Biologicals).

FIGURE 7. Structural elucidation of 5-cyanomethyl uridine. (A,C) High mass-accuracy mass spectra of the unknown ribonucleoside species (A) found in the purified *H. marismortui* $\text{tRNA}_2^{\text{lle}}$ U34 mutant and the 5-cyanomethyl-2'-deoxyuridine synthetic standard (C). The parent ions have m/z values of 284.0872 (A) and 268.0928 (C), and the deglycosylated base ions have identical m/z values of 152.0451 (A,C). (B,D) Pseudo-MS³ mass spectra of the unknown ribonucleoside species (B) and the 5-cyanomethyl-2'-deoxyuridine synthetic standard (D). Following in-source fragmentation of the parent ribonucleoside, the deglycosylated base ion was subjected to CID, with the product ions (numbered 1–5) in close agreement with the theoretical fragmentation pathway shown in panel E. The MRM chromatogram of the m/z 82→39 transition (species 5) is shown in the insets of B and D. (E) Proposed fragmentation pathway for 5-cyanomethyl uracil, with each numbered fragment along with its formula and exact mass.

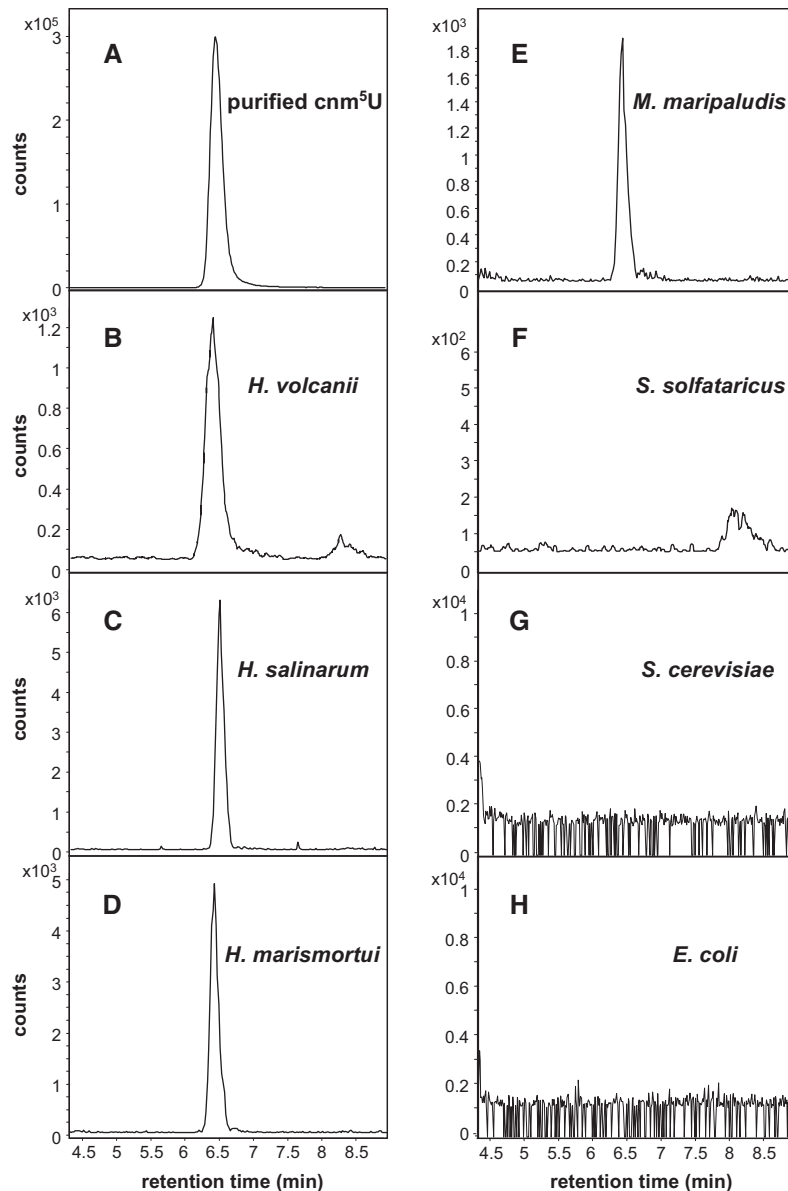


FIGURE 9. cm^5U in total tRNAs from haloarchaea, *M. maripaludis*, *S. solfataricus*, *S. cerevisiae*, and *E. coli*. Nucleoside detection was performed, either by monitoring m/z 284.2 \rightarrow 125 transition on an Agilent 6510 triple-quadrupole mass spectrometer (A–F) or by extracted ion chromatogram for the nucleoside m/z 284.2 on an Agilent 6520 QTOF mass spectrometer (G,H).

Polyacrylamide gel electrophoresis (PAGE) and Northern blot analysis of tRNA

Total RNA was extracted under acidic conditions using Trizol (Invitrogen) as described before (Köhler et al. 2008). RNA was re-suspended in 10 mM sodium acetate pH 5.0 and stored at -80°C . tRNAs were analyzed by acid urea PAGE (Varshney et al. 1991; Köhler and RajBhandary 2008) or native PAGE as indicated, followed by Northern blotting (Köhler and RajBhandary 2008). tRNAs were visualized by hybridization using ^{32}P -labeled DNA oligonucleotides according to standard procedures (Sambrook and Russell 2001). Oligonucleotides were 5'-end labeled with γ - ^{32}P -ATP (3000 Ci/mmol; PerkinElmer) using T4-PNK. North-

ern blots were analyzed by autoradiography followed by PhosphorImaging using Imagequant software.

Purification of overexpressed *H. marismortui* tRNA₂^{Ile} and tRNA₁^{Ile} (WT and U34 mutant) from *H. volcanii*

H. volcanii transformants were grown at 37°C to an OD_{600} of 3–3.5, and crude RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi 2006). Ribosomal RNA was removed by precipitation with 1 M LiCl yielding total tRNA. Purification of overexpressed isoleucine tRNAs was done as described earlier for tRNA₂^{Ile} (Suzuki and Suzuki 2007; Mandal et al. 2010), involving hybrid selection of the desired tRNA using a biotinylated DNA oligonucleotides immobilized to a streptavidin resin. The biotinylated oligonucleotide for tRNA₂^{Ile} purification was complementary to nucleotides 54–73 of the tRNA; the biotinylated oligonucleotide for tRNA₁^{Ile} purification was complementary to nucleotides 39–58 of the tRNA. Affinity purification was followed by electrophoresis of the enriched tRNA on native 10%–12.5% polyacrylamide gels. After elution from the gel and extensive dialysis against 5 mM ammonium acetate pH 5.5, the tRNA was concentrated by evaporation, precipitated with ethanol, and the precipitate was washed several times with 70% ethanol. Typically, about 4.0 A_{260} units of the U34 mutant of tRNA₂^{Ile} were obtained from approximately 1000 A_{260} of total tRNA; about 5.5 A_{260} units of the U34 mutant of tRNA₁^{Ile} were obtained from about 400 A_{260} of total tRNA. The purity of the tRNAs was assessed by PAGE, in vitro aminoacylation with isoleucine, and partial digestion of 5'- ^{32}P -labeled tRNAs with RNases T1, A, and U2.

In vitro aminoacylation of tRNA

One hundredth to 1.0 A_{260} units of tRNA were aminoacylated in vitro with L-isoleucine as described below using purified *E. coli* IleRS or S10, S30, or S100 extracts prepared from *H. volcanii*. Reaction mixtures using purified *E. coli* IleRS contained 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) pH 7.5, 10 mM MgCl_2 , 5 mM ATP, 0.1 $\mu\text{g}/\mu\text{L}$ BSA, 5 μM [^3H]-isoleucine (American Radiolabeled Chemicals), and 0.05 μM of IleRS. Alternatively, in vitro aminoacylations were carried using S10, S30, or S100 extracts from *H. volcanii* in a reaction containing 10 mM Hepes pH 7.5, 2.5 M KCl, 50 mM $\text{Mg}(\text{OAc})_2$, 5 mM ATP, 5 μM [^3H]-isoleucine, and ~ 0.5 $\mu\text{g}/\mu\text{L}$ extract. At various time points, aliquots were removed and analyzed by precipitation with TCA followed by liquid scintillation counting of TCA-precipitable counts.

Background (obtained from reactions run without tRNA) was subtracted from all values unless otherwise noted. Quantitative aminoacylation of purified *H. marismortui* isoleucine tRNAs for ribosome binding assays were carried out with purified *E. coli* IleRS; for this, reactions were allowed to proceed for 2 h, and inorganic pyrophosphatase was added at a final concentration of 0.04 units/ μ L. After aminoacylation, tRNAs were extracted with acid phenol and precipitated with ethanol.

Isolation of *H. marismortui* and *H. volcanii* ribosomes

Ribosomes from *H. marismortui* and *H. volcanii* were isolated as described (Mandal et al. 2010) using the basic procedure of Wittman and coworkers (Shevack et al. 1985) with minor modifications. Cells from a 2 L culture grown to midlog phase ($OD_{600} \sim 3.5$) were suspended in ice-cold 10 mM Hepes pH 7.5, 100 mM Mg(OAc)₂, 3.4 M KCl and 6 mM 2-mercaptoethanol ("high-salt ribosome buffer"), and lysed by French Press (Constant Cell Disruption System; 10,000 Psi, 2 passages). The ribosomes were then prepared as described, re-suspended in "high-salt ribosome buffer," divided into aliquots, quick frozen, and stored at -80°C .

Binding of aminoacylated tRNAs to ribosomes

The mRNA oligonucleotides (AUGAUA, AUGAUC, AUGAAU, AUGAUG, and AUGUUU) used in the ribosome binding experiments were stored at a stock concentration of 10 mM. The standard reaction (10 μ L) contained 50 mM Tris-HCl pH 7.5, 50 mM NH₄Cl, 5 mM Mg(OAc)₂, 3 mM 2-mercaptoethanol, 2.1 M KCl, 1.2 A₂₆₀ units of *H. marismortui* ribosomes, RNA oligonucleotides (0–1.5 mM), and ~ 1.6 pmol [³H]-labeled tRNA (10,000 cpm). Alternatively, reactions (20 μ L) contained 50 mM Hepes pH 7.5, 50 mM NH₄Cl, 50 mM Mg(OAc)₂, 3 mM 2-mercaptoethanol, 2.1 M KCl, 1 A₂₆₀ unit of *H. volcanii* ribosomes, RNA oligonucleotides (0–1.5 mM), and ~ 1.6 pmol [³H]-labeled tRNA (10,000 cpm). Incubation was at room temperature for 1 h. The reaction was stopped by adding 0.5 mL wash buffer [50 mM Tris-HCl or Hepes pH 7.5, 50 mM NH₄Cl, 5 mM Mg(OAc)₂, 2.1 M KCl], and the mixture was filtered through a nitrocellulose filter (HA 0.45 μ m, Millipore) prewashed with the same buffer. The filters were washed several times with wash buffer, dried, and counted for radioactivity in a liquid scintillation counter.

5'-³²P-end labeling and partial digestion of tRNA

tRNA (0.01 A₂₆₀) was dephosphorylated with 1 unit of calf intestinal phosphatase in a 50- μ L reaction containing 50 mM Tris-HCl pH 7.9 and 1 mM EDTA. Before adding enzyme, the mixture was incubated for 2 min at 65°C and then for 5 min at 37°C. After dephosphorylation for 30 min at 37°C, the reaction mixture was incubated with 5 mM nitrilotriacetic acid (Sigma) for 30 min at room temperature, and the phosphatase was inactivated by heat for 2 min at 65°C. Dephosphorylated tRNAs were labeled with ³²P at the 5' terminus in a 50- μ L reaction containing 70 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol (DTT), 10 pmol of γ -[³²P]-ATP, and 20 U of T4-PNK. After 30 min of incubation at 37°C, the ³²P-labeled tRNA was precipitated with a 2.5 volume of ethanol, 0.2 M ammonium acetate pH 5.2, and 10 μ g glycogen (Ambion). The precipitate

was washed with 70% ethanol. ³²P-labeled tRNA was separated by electrophoresis on a 6% denaturing polyacrylamide gel, and the band corresponding to the full-length tRNA was excised and eluted in 10 mM Tris-HCl pH 7.5 and 5 mM MgCl₂. The eluted radiolabeled tRNA was further precipitated and washed as above and then dissolved in sterile water.

For partial alkali hydrolysis and partial RNase digestions, 20,000 cpm of ³²P-labeled tRNA and 0.01 A₂₆₀ units of total *H. marismortui* tRNA were used in reaction volumes of 7.5–10 μ L. The partial alkali hydrolysis reaction was in 33 mM Na₂CO₃/NaHCO₃ buffer pH 9.2 for 2–3 min at 95°C. The partial RNase T1 digestion was in 50 mM Tris-HCl pH 7.5, 7 M urea and 5–10 U of RNase T1 and incubation was for 10 min at 50°C. Partial digestion with RNase U2 (0.002 unit) was in 50 mM ammonium acetate pH 4.5 for 5 min at 37°C. Partial digestion with RNase A (0.002–0.005 unit) was in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA for 5 min at 37°C. After the incubation, samples were quick frozen, lyophilized to dryness, and then dissolved in formamide loading buffer. The samples were separated by electrophoresis on a 10% denaturing polyacrylamide gel; gels were dried and then used for autoradiography.

Nuclease P1 digests of 5'-³²P-end labeled fragments of tRNA

Purified tRNAs (0.01 A₂₆₀) were partially hydrolyzed with alkali as described above. The mixture was evaporated to dryness, and the residue was dissolved in 10 μ L water. Digested tRNA (0.001 A₂₆₀) was incubated with 5 units of T4-PNK in a 20- μ L reaction containing 70 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 5 mM DTT, and 2.5 pmol γ -[³²P]-ATP for 5 min at 37°C. The 5'-³²P-labeled fragments were separated by 10% denaturing PAGE, and bands corresponding to the anticodon base U34 (for mutant tRNA₂^{Ile} and tRNA₁^{Ile}) and bases preceding and following U34 were eluted with buffer A (20 mM Tris-HCl pH 7.5, 10 mM MgCl₂), precipitated with ethanol, washed, dissolved in sterile water, and digested with nuclease P1 in 50 mM ammonium acetate buffer pH 5.0 overnight at 37°C. The incubation mixtures were quick frozen on dry ice and lyophilized under vacuum. Nucleotides were dissolved in sterile water and lyophilized to dryness three times to remove any ammonium acetate prior to thin layer chromatography.

Thin layer chromatography

Cellulose F plates (20 \times 20 cm) with fluorescent background used for two-dimensional thin layer chromatography (2D-TLC) analyses were obtained from Merck. The solvent system for the first and second dimension were as follows: (1) isobutyric acid:concentrated ammonia:water (66:1:33); and (2) isopropanol:concentrated HCl:water (70:15:15). The first dimension was run in Solvent 1 for ~ 16 h; the plates were then dried overnight. The second dimension was run in Solvent 2 for ~ 28 h. Plates were dried and analyzed by autoradiography. Nonradioactive nucleotides were used as markers and visualized by UV shadowing.

Enzymatic hydrolysis of tRNA and HPLC analysis of modified ribonucleosides

Purified *H. marismortui* tRNA₂^{Ile} and tRNA₁^{Ile} (WT and U34 mutants) and total tRNA from *H. volcanii*, *H. salinarum*, *H.*

marismortui, *M. maripaludis*, *S. solfataricus*, *S. cerevisiae*, and *E. coli* were digested to nucleosides using nuclease P1, snake venom phosphodiesterase I, and antarctic phosphatase as described in detail by Crain (Crain 1990). After digestion, nucleosides were passed through a centrifugal filter (Amicon Ultra; 10K) for removal of enzymes.

Nucleosides were then separated by HPLC using an Agilent 1100 HPLC and a Thermo Scientific Hypersil GOLD aQ reverse-phase column (150 × 2.1 mm, 3 μm) eluted with the following gradient of water to acetonitrile containing 8 mM ammonium acetate at a flow rate of 0.3 mL/min and 36°C: 0–18 min, 0%; 18–23 min, 0%–1%; 23–28 min, 1%–6%; 28–30 min, 6%; 30–40 min, 6%–100%; 40–50 min, 100%; the HPLC eluent was monitored at 260 nm using a diode-array detector.

Liquid chromatography-coupled mass spectrometric analysis of ribonucleosides

Ribonucleosides identified by HPLC were structurally characterized by LC-MS analysis. Initial analysis of the unknown ribonucleoside was performed using an Agilent 1290 UPLC equipped with diode array detectors and a Thermo Scientific Hypersil GOLD aQ C18 reverse-phase column (100 × 1 mm, 1.9 μm particle size) that was coupled to an Agilent 6510 QTOF high resolution mass spectrometer with an electrospray ionization source operated in positive ion mode with the following parameters: drying gas temperature, 325°C; drying gas flow, 8 L/min; nebulizer, 25 psi; and capillary voltage, 3500 V. The QTOF was operated in MS mode, with 140 V fragmentor voltage and with mass range m/z 100–1000. HPLC resolution was performed with the same gradient as before of water to acetonitrile at a flow rate of 70 μL/min and 36°C: 0–18 min, 0%; 18–23 min, 0%–1%; 23–28 min, 1%–6%; 28–30 min, 6%; 30–40 min, 6%–100%; 40–50 min, 100%. The unknown species with m/z 284.0872 eluted at 6.6 min.

Collision induced dissociation (CID) experiments were performed using an Agilent 1200 HPLC with the same Thermo Scientific Hypersil GOLD aQ reverse-phase column coupled to the QTOF mass spectrometer. HPLC resolution was performed isocratically with 8 mM ammonium acetate at a flow rate of 70 μL/min for 20 min and 36°C. The unknown species with m/z 284.0872 eluted at 4.27 min and 5-cyanomethyl-2'-deoxyuridine eluted at 3.56 min. The initial CID analysis of 5-cyanomethyl-2'-deoxyuridine and the unknown molecular species was performed in targeted MS/MS mode on m/z 268.0928 or m/z 284.08771, respectively, and a fragmentor voltage of 140 V or 90 V, respectively, with collision energies of 5 V, 10 V, or 15 V used to optimize product ion formation. In-source fragmentation and subsequent CID analysis of protonated base was used to perform pseudo-MS³ analysis of both 5-cyanomethyl-2'-deoxyuridine and the unknown molecular species. In this analysis, a fragmentor voltage 300 V was used in targeted MS/MS on the m/z 152.04545 fragment of 5-cyanomethyl-2'-deoxyuridine, with collision energies of 5 V, 10 V, or 15 V. For the unknown species, a fragmentor voltage of 160 V was used for targeted MS/MS on the m/z 152.04545 fragment of the unknown ribonucleoside.

Subsequent fragmentation analysis by triple-quadrupole (QqQ) mass spectrometry was performed by MRM analysis using an Agilent 1100 HPLC with a Thermo Scientific Hypersil GOLD aQ column (150 × 2.1 mm, 3 μm particle size) coupled to an Agilent

6410 QqQ mass spectrometer. For analysis of 5-cyanomethyl-2'-deoxyuridine and the purified unknown species, ribonucleosides were eluted isocratically with 8 mM ammonium acetate at a flow rate of 0.2 mL/min for 20 min and 36°C. The QqQ mass spectrometer with an electrospray ionization source was operated in positive ion mode with the following parameters: drying gas temperature, 325°C; drying gas flow, 8 L/min; nebulizer, 30 psi; and capillary voltage, 4000 V. The first and third quadrupoles (Q1 and Q3) were set to unit resolution and the monitored ion transitions. The m/z of the transmitted parent ion, m/z of the monitored product ion, fragmentor voltage, and collision energy, respectively, for 5-cyanomethyl-2'-deoxyuridine were as follows: 268.2→152.1, 90 V, 5 V; 152.1→125.1, 140 V, 5 V; 125.1→82.1, 150 V, 10 V; 82.1→39, 160 V, 15 V. For cnm^5U , the parameters were: 284.2→152.1, 90 V, 5 V; 152.1→125.1, 140 V, 5 V; 125.1→82.1, 150 V, 10 V; 82.1→39, 170 V, 15 V. All MRM produced peaks at the same retention time (transition 82.1→39 shown in Fig. 7B, inset), suggesting they are from the same parent compound. Detection of cnm^5U in purified tRNA or total tRNA was achieved by resolving enzymatic hydrolysates on the reversed-phase HPLC column with a gradient of water to acetonitrile containing 8 mM ammonium acetate at a flow rate of 0.2 mL/min and 36°C: 0–15 min, 0%; 15–16 min, 0%–100%; 16–20 min, 100%; 20–21 min, 100%–0%. The QqQ mass spectrometer was operated in positive ion mode with the following parameters: gas temperature, 325°C; gas flow, 10 L/min; nebulizer, 20 psi; and capillary voltage, 4000 V. The m/z of the transmitted parent ion, m/z of the monitored product ion, fragmentor voltage, and collision energy were as follows: 284.2→125.1, 110 V, 15 V; 152.1→125.1, 140 V, 10 V; 152.1→82.1, 140 V, 15 V.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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